APPLIED MICROBIOLOGY, May 1967, p. 590-593

Vol. 15, No. 3
Printed in U.S.A.

Enhanced Toxicity for Mice of Pertussis Vaccines When Preserved with Merthiolate

E. A. NELSON AND R. Y. GOTTSHALL

Division of Biologic Products, Bureau of Laboratories, Michigan Department of Public Health, Lansing, Michigan 48914

Received for publication 16 December 1966

Pertussis vaccines preserved with 0.01% Merthiolate are more toxic for mice than unpreserved vaccines prepared from the same parent concentrate and containing the same number of organisms. The toxicities of both Merthiolate (0.01%)-preserved and unpreserved vaccines increased when the number of organisms injected was increased. An increase in mortality was observed when Merthiolate was injected separately, before or after an unpreserved saline suspension of pertussis vaccine.

In the preparation of pertussis vaccine, the bacteria are separated from the liquid or solid medium on which they were grown and are killed by adding Merthiolate (final concentration 1:5,000) and storing at 5 C or by heating at 56 C with or without formaldehyde. The killed suspensions of pertussis organisms are often toxic, but the toxicity of most lots declines to a low level during storage. Toxicity is measured by injecting mice intra-abdominally with a suspension of the vaccine diluted in physiological saline and observing weight change and mortality.

In the course of preparing and testing pertussis vaccines, we have found that they are less toxic for mice when diluted in saline than when diluted in saline with Merthiolate at a final concentration of 1:10,000. This preservative is widely used for preserving pertussis vaccine, whether the vaccine is employed as a single antigen or is combined with diphtheria or tetanus toxoids. Merthiolate is not very toxic for mice. Powell and Jamieson (8) reported that mice are able to tolerate 1 to 2 mg of Merthiolate (intravenously) without adverse effect.

Gardner and Pittman (2) found the potency of pertussis vaccines more stable when the vaccines were preserved with Merthiolate than when they were left unpreserved. This is also observed in some of the data presented by Olson, Eldering, and Graham (6). So far, no reports have come to our attention of adverse toxic reactions in mice resulting from the combination of Merthiolate and pertussis vaccine. This report presents several aspects of the comparative toxicity of Merthiolate-preserved and unpreserved pertussis vaccines.

MATERIALS AND METHODS

Pertussis vaccines A, B, and C (Table 1) were prepared on Bordet-Gengou medium with the use of 1:5,000 Merthiolate to kill the organisms (10). Vaccine D was prepared by growing the bacteria on Bordet-Gengou medium, but the organisms were killed by heating at 56 C for 60 min in the presence of 0.1% formaldehyde solution; 1:40,000 benzethonium chloride (Hyamine 1622, Rohm & Haas Co., Philadelphia, Pa.) was then added. Vaccine E was prepared in the medium described by Cohen and Wheeler (1), and vaccines F, G, and H were prepared on the medium described by Mishulow, Sharpe, and Cohen (4). The bacteria in these vaccines (E, F, G, H) were killed by heating at 56 C for 30 min, and were preserved with 0.01% Merthiolate.

Merthiolate (thimerosal) was obtained from Eli Lilly & Co., Indianapolis, Ind. A 10% stock solution was prepared as described on the circular accompanying the preservative.

The mice used for the toxicity test were a Swiss Webster strain raised at the Michigan Department of Public Health Laboratories. The test was performed as outlined in the 1961 revision of the Minimum Requirements for Pertussis Vaccine (*see* 2). Ten male or ten female mice, each weighing 14 to 16 g, were used for a single test. For any one vaccine, however, the toxicity tests were often repeated, and the results are reported as combinations of separate tests. The group weight of the mice was obtained 2 to 3 hr before injection. The vaccines injected contained 10 to 10.5 opacity units (OU) in 0.5 or 0.7 ml, and were administered intra-abdominally. For controls, groups of 10 mice were injected with either 0.5 ml of saline or 0.7 ml of saline containing 0.01% Merthiolate. The animals were kept for 7 days after injection of the vaccine or saline; food and water were available constantly. As suggested in the official test, a vaccine was

TABLE 1. *Toxicity of pertussis vaccines diluted in saline or in saline and Merthiolate (0.01%)*

Vaccine	Growth medium	Method of inactivation	Merthiolate injected per mouse	Toxicity test				
				Organisms injected (opacity units)	Vol injected	Gain in wt	Mortality	No. and sex of mice
			mg		*ml*	*g*	%	
A	Bordet-Gengou	Storage with Merthiolate	0.005	10.0	0.5	3.4	2.0	50 F
			0.070	10.5	0.7	2.9	9.2	70 M; 60 F
B	Bordet-Gengou	Storage with Merthiolate	0.005	10.0	0.5	3.6	3.3	120 M; 60 F
B_1			0.070	10.5	0.7	2.3	0.9	80 M; 10 F
B_2			0.070	10.5	0.7	2.9	25.0	20 M
C	Bordet-Gengou	Storage with Merthiolate	0.0045	10.0	0.5	3.5	0.0	30 M
			0.070	10.5	0.7	3.7	15.5	90 M
D	Bordet-Gengou	Heat (56 C) and 0.1% HCHO[a]	0.000	10.0	0.5	4.1	7.5	30 M
			0.070	10.5	0.7	3.6	12.0	40 M; 10 F
E	Cohen-Wheeler	Heat (56 C)	0.004	10.0	0.5	4.3	0.0	40 F
			0.050	10.0	0.5	3.6	13.3	30 F
F	MSC[b]	Heat (56 C)	0.004	10.0	0.5	5.3	2.5	40 F
			0.050	10.0	0.5	3.9	2.5	40 F
G	MSC	Heat (56 C)	0.004	10.0	0.5	4.9	0.0	40 F
			0.050	10.0	0.5	5.2	10.0	30 F
H	MSC	Heat (56 C)	0.004	10.0	0.5	4.8	0.0	10 M; 10 F
			0.050	10.0	0.5	3.6	25.0	10 M; 10 F
Controls			0.000	None	0.5	5.9	0.0	120 M; 50 F
			0.070	None	0.7	5.6	0.0	10 M; 20 F

[a] Formaldehyde.
[b] Mishulow-Sharpe-Cohen.

accepted as free from toxicity if: (i) at the end of 72 hr the group weight was no less than the initial weight, (ii) the average gain in weight 7 days after injection was 3.0 g or more per mouse, and (iii) the mortality at 7 days did not exceed 5%.

Preserved and unpreserved vaccines A, B, C, and D were not tested concurrently. With these vaccines, saline dilutions of the concentrated parent suspensions (originally containing 300 to 500 OU per ml) were tested for toxicity at intervals after the bacteria were killed. These parent vaccines were diluted for the toxicity test so that the mouse dose of 0.5 ml contained 10 OU of vaccine. This dose contained 0.005 mg or less of residual Merthiolate. When the vaccines were sufficiently detoxified by storage so that they passed the official toxicity test, a portion of the remaining concentrated parent suspension was diluted in saline to 15 OU per ml and preserved with 0.01% Methiolate. For the toxicity test, each mouse received 10.5 OU of the preserved vaccine intra-abdominally.

Preserved and unpreserved vaccines E, F, G, and H were tested concurrently. The mouse dose for the toxicity test was 10 OU in a volume of 0.5 ml.

RESULTS

The comparative toxicities of the eight pertussis vaccines diluted in saline alone and in saline-Merthiolate (0.01%) are given in Table 1. With all vaccines except B1, the mortality was greater with those vaccines diluted in saline and preserved with 0.01% Merthiolate than in those diluted in saline and not preserved. In vaccine F, the mortality was the same with both vaccines.

With all of the vaccines except C and G, the mice gained more weight when they received vaccine in saline than when they received vaccine diluted in saline and Merthiolate. The control mice, which received only saline or saline and Merthiolate, gained more weight than those that received pertussis vaccine. The greater toxicity of pertussis vaccines diluted in saline and preserved with Merthiolate was evident with all of the vaccines tested, regardless of the method of preparation. Only one preserved vaccine (F) would have

TABLE 2. *Effect of number of organisms injected on toxicity of unpreserved pertussis vaccine and on vaccine preserved with 0.01% Merthiolate*

Preservative added to diluted vaccine	Dose per mouse (opacity units)	No. and sex of mice	Merthiolate injected	Outcome		
				D/n[a]	Mortality	Avg wt gain
			mg		%	*g*
None	5	10 M; 10 F	0.002	0/20	0	5.2
	10	10 M; 10 F	0.004	0/20	0	4.9
	20	10 M; 10 F	0.008	2/20	10	2.7
Merthiolate to 0.01%	1	10 M; 10 F	0.05	0/20	0	5.2
	5	20 M; 10 F	0.05	3/30	10	5.1
	10	20 M; 10 F	0.05	5/30	17	3.8
	20	10 M	0.05	3/10	30	1.7

[a] Deaths/number tested.

TABLE 3. *Effect of the toxicity response in mice of injection of Merthiolate before, with, and after pertussis vaccine*[a]

Time Merthiolate was injected	Quantity of Merthiolate injected	No. and sex of mice	Toxicity		
			D/n[b]	Mortality	Avg wt gain on given day after first injection
	mg			%	
24 hr before vaccine.....	0.05	40 M	3/40	7.5	5.1 g, 8 days
With vaccine (control)..	0.05	30 M	4/30	13.3	4.5 g, 7 days
24 hr after vaccine.....	0.05	40 M	2/40	5.0	4.7 g, 8 days
48 hr after vaccine.....	0.05	30 M	1/30	3.3	4.8 g, 9 days
Unpreserved vaccine (control).....	0.005	50 M	1/50	2.0	3.8 g, 7 days

[a] Dose of vaccine: 10 opacity units in 0.5 ml of saline.
[b] Deaths/number tested.

passed the official toxicity test for pertussis vaccine; with the other vaccines, either the average gain in weight was less than 3.0 g per mouse, or the mortality was greater than 5%. Statistical analysis (*t* test) of the mortalities after injection of vaccines E, F, G, and H showed a significant difference between the preserved and unpreserved materials. The probability that this difference occurred by chance is 0.005 or less.

Table 2 shows the effect of the concentration of organisms injected on toxicity of preserved and unpreserved vaccine. Dilutions of concentrated pertussis vaccine (vaccine B) were prepared in saline alone and in saline with Merthiolate (0.01% concentration). Groups of 10 mice were inoculated with 5, 10, and 20 OU per mouse of unpreserved vaccine and 1, 5, 10, and 20 OU of Merthiolate-preserved vaccine. An increase in the number of pertussis organisms per mouse dose resulted in an increase in toxicity. This effect was most apparent with the vaccines containing the 0.01% concentration of Merthiolate. The highest mortality and least gain in weight occurred when Merthiolate-preserved vaccine was injected in a dose of 20 OU.

The toxicity test results after administration of 0.05 mg of Merthiolate (0.5 ml of 1:10,000 Merthiolate) in saline to groups of mice 24 hr before, and 24 and 48 hr after, the injection of 10 OU of unpreserved vaccine are recorded in Table 3. For controls, three groups of 10 mice were injected with 10 OU of a vaccine preserved with 0.01% Merthiolate, and five groups of 10 mice received 10 OU of the same vaccine unpreserved. The data indicate that mortality was greater when Merthiolate was injected 24 hr before, or 24 or 48 hr after, administration of a saline suspension of unpreserved pertussis vaccine than when an unpreserved suspension was injected alone. Mortality was greatest, however, when Merthiolate was injected with the vaccine.

DISCUSSION

The greater toxicity in mice of Merthiolate-preserved pertussis vaccine compared with unpreserved vaccine may be due to: (i) reactivation by Merthiolate of an atoxic bacterial toxin, (ii) lysis of bacterial cells by Merthiolate with liberation of an endotoxin, (iii) increase in susceptibility of the mice to the toxicity of Merthiolate induced by pertussis vaccine, or (iv) increase in susceptibility to the toxicity of pertussis vaccine induced by Merthiolate. In all of the experiments, deaths were distributed throughout the 7-day observation period, and the times of death gave no clue as to whether the vaccine injected was slightly toxic or was one which was atoxic with the toxicity being due to addition of Merthiolate.

Since increased susceptibility of mice toward such widely different physical and chemical agents as histamine (7, 9), reduced atmospheric pressure (3), cold stress (5), and ozone (E. J. Fairchild, G. A. Bobb, and G. E. Thompson, Federation Proc. **25**:692, 1966) follows injection of pertussis vaccine, it would not be surprising if injection of this vaccine influenced the susceptibility of the mouse toward a mercurial preservative. The toxicity in mice of final lots of preserved vaccine prepared from concentrated vaccines which were atoxic or only slightly toxic has been observed in other laboratories and has been the source of much speculation.

We have no data to suggest that Merthiolate-preserved pertussis vaccines which show a greater toxicity in mice than unpreserved vaccines also have a greater toxicity in man.

ACKNOWLEDGMENT

We are grateful to George Van Amburg for making the statistical analyses.

LITERATURE CITED

1. COHEN, S. M., AND M. W. WHEELER. 1946. Pertussis vaccine prepared with Phase I cultures grown in fluid medium. Am. J. Public Health **36**:871.
2. GARDNER, R. A., AND M. PITTMAN. 1965. Relative stability of pertussis vaccine preserved with Merthiolate, benzethonium chloride, or the parabens. Appl. Microbiol. **13**:564–569.
3. KIND, L. S., AND R. H. GADSDEN. 1953. Adrenal function tests in mice sensitized to histamine with *Hemophilus pertussis* vaccine. Proc. Soc. Exptl. Biol. Med. **84**:373–375.
4. MISHULOW, L., L. S. SHARPE, AND L. L. COHEN. 1953. Beef-heart charcoal agar for the preparation of pertussis vaccines. Am. J. Public Health **43**:1466–1472.
5. MUNOZ, J., AND L. F. SCHUCHARDT. 1957. Effect of *H. pertussis* on sensitivity of mice to cold stress. Proc. Soc. Exptl. Biol. Med. **94**:186–190.
6. OLSON, B. H., G. ELDERING, AND B. GRAHAM. 1964. Stabilization of pertussis vaccine in the presence of benzethonium chloride. J. Bacteriol. **87**:543–546.
7. PARFENTJEV, I. A., M. A. GOODLINE, AND M. E. VIRION. 1947. A study of sensitivity to Hemophilus pertussis in laboratory animals. II. Hemophilus pertussis allergen and its assay on laboratory animals. J. Bacteriol. **53**:603–611.
8. POWELL, H. M., AND W. A. JAMIESON. 1931. Merthiolate (Thimerosal, Lilly) as a germicide. Am. J. Hyg. **13**:296–310.
9. ROWEN, M., W. W. MOOS, AND M. SAMTER. 1955. Increased radiation sensitivity of pertussis-vaccinated mice. Proc. Soc. Exptl. Biol. Med. **88**:548–550.
10. WORLD HEALTH ORGANIZATION. 1953. Diphtheria and pertussis vaccination. Report of a conference of heads of laboratories producing diphtheria and pertussis vaccines. World Health Organ. Tech. Rept. Ser. No. 61, p. 69–82.